INFLUENCE OF NITRATE REDUCTASE ACTIVITY OF *STAPHYLOCOCCUS CARNOSUS* ON THE FORMATION OF NITRATE AND NITRITE IN RAW HAM

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The influence of nitrate reductase activity of *Staphylococcus carnosus* starter cultures on the formation of nitrite and nitrate in raw ham was investigated. Therefore brine either with nitrite or nitrate was injected with or without *S. carnosus* starter cultures to produce raw hams. Microbiological, chemical and sensory analysis were done over process and storage. During process and storage, the level of inoculated Staphylococci remained constantly on 6.5 log cfu/g meat whereas the intrinsic microflora of the hams without starter cultures started growing after day 14. The raw ham with the high nitrate reductase active *S. carnosus* LTH 7036 showed the highest decrease in nitrate and high nitrite concentration in the legal level. The hams cured with nitrate and without starter culture or with S. carnosus LTH 3838 showed higher residual nitrate levels and lower nitrite concentrations after 34 days. Therefore, the selection of starter cultures due to their nitrate reductase activity plays an important role in the formation of reactive intermediate curing compounds and color formation in raw ham.

Key Words: raw ham curing, specific enzyme activities, starter cultures

I. INTRODUCTION

Meat curing is an old and traditional technique to preserve meat from spoilage [1, 2]. Nowadays not merely preservation of meat is on the focus of curing meat, but rather the aromatic taste and convincing appearance of meat products. Therefore the typical red color of raw cured products is one of the main aims of curing meat products [1, 2]. Nitrite is the agents that is responsible for the formation of nitric oxide that react with the meat pigment Myoglobin to the typical reddish Nitrosylmyoglobin [2]. Moreover, nitrite added directly or produced by microorganism with nitrate reductase activity have more benefits in raw meat products, as suppression of growth of anaerobic bacteria like *Clostridium botulinum*, flavor development and its antioxidative activity [1, 2]. Nevertheless the usage of nitrite and nitrate is strictly regulated by government because of the formation of N-nitrosamines from reaction of nitrite with amino acids and amines [2]. In Europe the maximum amount of sodium nitrite for raw ham is 50 mg/kg and potassium nitrate is 250 mg/kg in the final product (Regulation (EC) No. 1333/2008). Starter cultures with nitrate reductase activity such as Staphylococci could be used to control the fermentation and reduction of nitrate. Also when only nitrite is used because of the disproportionation of nitrite to NO and nitrate [3]. In particular the raw ham production without a usage of lactic acid bacteria, no acceleration of the curing process could be achieved [3]. Therefore the nitrate reductase activity is a important factor for the selection of starter cultures in raw ham fermentation.

The aim of this study is the investigation of the influence of the nitrate reductase activity of selected *Staphylococcus carnosus* starter cultures on the formation of nitrate and nitrite in raw hams due to color formation and product quality.

II. MATERIALS AND METHODS

II.1 Selection and Preparation of Starter Cultures

Selection of starter cultures was based on preliminary experiments on the physiological properties of *S. carnosus* strains [4]. *S. carnosus* LTH 3838 was chosen because of its low nitrate reductase activity and its high proteolytic activity and *S. carnosus* LTH 7036 was used because of its high nitrate reductase activity. The bacterial

strains were grown in 100 mL Standard 1 nutrient broth (Merck, Darmstadt Germany) at 37 °C for 24 h at 180 rpm in incubator shaker (Innova 42, Eppendorf, Hamburg, Germany). Cells were concentrated by centrifugation (2500 x g, 10 min, 4 °C) of 12 x 40 mL, re-suspended in 1 mL 0.9 % NaCl solution (Carl Roth, Karlsruhe, Germany) and combined. The combined cells were washed twice in 0.9 % NaCl solution and final re-suspended in 1 mL 0.9 % NaCl solution. In diluted samples, the specific optical density at 600 nm was measured to get a cell concentration of approx. 10^{11} cfu/mL.

II.2 Raw Ham Production

Fresh pork loin (pH: 5.46 ± 0.05; *Musculus longissimus dorsi*) was obtained from a local central market (MEGA, Stuttgart, Germany). For all batches 30 L of brine was made with 10 % curing salt either with nitrite or nitrate and done in duplicate. Nitrate curing salt was produced by using 13.18 g KNO_3 (Gewürzmüller, Korntal-Münchingen, Germany) per kg NaCl. 4 batches were made by using either nitrite or nitrate as curing agents with or without one starter culture in brine for injection of hams. For the first batch (nitrate control), brine was made with nitrate and without starter culture. The second batch (nitrite control) contained a commercial 0.9 % sodium nitrite curing salt (Südsalz, Heilbronn, Germany) without starter culture. The brine with *S. carnosus* LTH 3838 (batch 3) was produced by using nitrate curing salt and 1 mL cell suspension with approx. 10^{11} cfu/mL. Batch 4 contained *S. carnosus* LTH 7036 and nitrate curing salt. All hams were injected using a multi needle injector 105 MC2 R (Günther, Dieburg, Germany) with the following setting: 105 needles (2 mm diameter, 2 x 0.8 mm hole size), 6 bar injection pressure , triple and two-way injection to get a injection weight of 7 - 8 %. After injection a dry curing step was applied to get a curing salt content of 40 g per kg fresh meat and stored for 7 days at 5 °C. Drying and mild smoking (at 24 °C) took place in the climatic chamber Air Master UK-1800 BE (Reich, Urbach, Germany) for 7 days with temperatures of 10 to 15 °C with a relative humidity gradient from 85 to 75 %. Afterwards hams were stored at 15 °C at 75 % rel. humidity till they reached a final weight loss of 27 % (26.76 ± 0.9 %) and vacuum packed. The final vacuum packed hams were stored till day 34 at 15 °C. All analyses during process and storage were done in duplicate.

II.3 Microbiology Analysis

Microbiological samples were taken with sterile knife. Approx. 10 g sample was weighted in a stomacher bag (VWR International, Darmstadt Germany) and diluted 1:10 gravimetrical in peptone (Carl Roth, Karlsruhe, Germany) and homogenized using stomacher (90s, IUL instruments, Barcelona, Spain). Samples were diluted and 50 or 100 µL were plated pour or spiral using automated spiral plater (Don Whitley Scientific Limited, West Yorkshire, United Kingdom). For the detection of the total viable count Plate Count Agar (AppliChem, Darmstadt, Germany) was incubated for 48 h at 37 °C. Staphylococci were detected on Baird Parker Agar (Carl Roth, Karlsruhe, Germany) incubated for 72 h at 37 °C. Colony forming units (cfu) were measured using aCOLyte colony counting device (Synbiosis, Cambridge, United Kingdom).

II.4 Nitrite and Nitrate Analysis

The analysis was carried out in line to [5] with slide modification in sample preparation. 15 g sample was cut in small cubes and weighted in glasses added with 10 mL borax solution (Disodiumtetraborate, Merck, Darmstadt, Germany) and stored at 5 °C till analysis. The sample in borax solution was transferred with 80 mL hot distilled water in Erlenmeyer flask and homogenized using Ultra Turrax (1 min, 13500 rpm; SilentCrusher, Heidolph Instruments, Schwabach, Germany). The next steps followed method of Schoch *et al.* [5]. A HPLC System HP Agilent Series 1100 (Santa Clara, USA) with UV detector at 224 nm with PRP-X100 column (Hamilton, Reno, USA) and software ChemStation (Agilent, Santa Clara, USA) were used. Calibration curves of nitrite and nitrate with 0.2 – 2 mg/L were established. The integration was manually done for nitrite at 2.2 min and nitrate at 3.8 min.

II.5 Sensory Analysis

The sensory analysis was done on day 20 and 34, after storage at 15°C. A panel of 20 assessors rated the red color, aromatic odor and aromatic taste of the 3 nitrate ham batches in comparison to the nitrite control as reference standard. A scale from

0 (less) to 10 (high) was used where the reference standard was set to 5.

III. RESULTS AND DISCUSSION

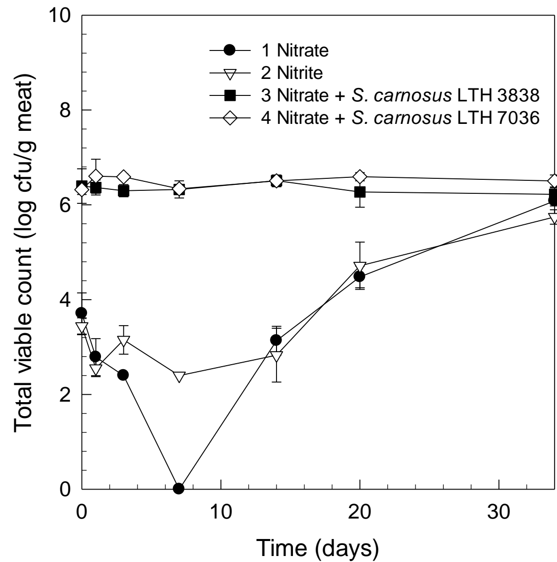
The inoculation level of raw ham is influenced by the microbiological load of the brine and the injected amount of brine. Table 1 summarizes the results of the microbiology analysis of the produced brines and the injection weight. The brines of the control raw hams had low total viable and staphylococcal counts under 2.3 log cfu/mL. The brines with starter cultures contained either 7.44 to 7.68 log cfu/mL total viable counts or staphylococcal counts of 7.02 to 6.89 log cfu/mL. The injection of brine varied slightly between the batches with a mean injection weight of 7.81 ± 1.32 % (Table 1).

During process and storage of the inoculated raw ham samples (batch 3 and 4), the total viable counts remained constantly on a level of 6.5 log cfu/g meat (Figure 1). No growth of the intrinsic microflora in the control batches (1 and 2) could be observed during curing (day 7), because of the low total viable counts in raw meats (data not shown) and brines. Moreover, the low temperature of 5 °C during curing prevented microbiological growth. During drying and ripening the temperature was increased to 10 °C and 15 °C and the intrinsic microflora of the control batches grew. At the end of the storage after 34 days, the total viable counts of control batches 1 and 2 raised to the counts of the inoculated raw hams. Thereby, the choice of curing agents did not seem to influence the growth of intrinsic microflora in cured raw hams without addition of starter culture.

Table 1: Summary of the injection parameters of the ham batches with total viable count, staphylococcal count in brine and the injection weight of each batch (mean ± standard deviation)

Batch	Total viable count in brine (log cfu/mL)	Staphylococcal count in brine (log cfu/mL)	Injection weight (%)
1 Nitrate Control	< 2.30	< 2.30	7.07 ± 0.49
2 Nitrite Control	< 2.30	< 2.30	8.68 ± 1.51
3 Nitrate + *S. carnosus* LTH 3838	7.68 ± 0.08	7.02 ± 0.14	7.79 ± 1.96
4 Nitrate + *S. carnosus* LTH 7036	7.44 ± 0.22	6.89 ± 0.13	7.71 ± 0.79

Figure 1: Growth of microorganisms during process and storage of all raw ham batches.

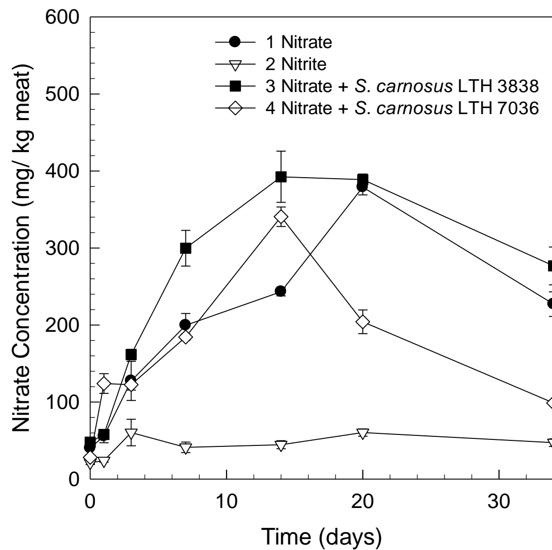


The presence of starter cultures or intrinsic microflora influences the trend of nitrate and nitrite over process and storage of the raw hams. Figure 2 a and b describe the nitrate and nitrite concentrations of the raw ham batches over time. Till day 14 the concentration of nitrate increased for all samples cured with nitrate (batch 1, 3, 4) and the nitrite concentration remained zero. This could be explained by the dry curing step and the diffusion of nitrate and salt to the center of the raw hams. While and after drying and ripening the different nitrate reductase activities of the strains have an effect. Batch 4 with *S. carnosus* LTH 7036 with the highest nitrate reductase activity showed the highest decrease in nitrate concentration and the highest concentration of nitrite under the legal maximum quantity of 50 mg/kg. In contrast to batch 3 with *S. carnosus* LTH 3838 with the lowest nitrate reductase activity showed the highest nitrate (in excess of legal maximum quantity of 250 mg/kg) and low nitrite concentration after a storage till day 34. The nitrate control (batch 1) without starter cultures showed a decrease in nitrate after 20 days and an increase in nitrite concentration when the growth of the intrinsic microflora started. The same trend over time for nitrite could be observed for the

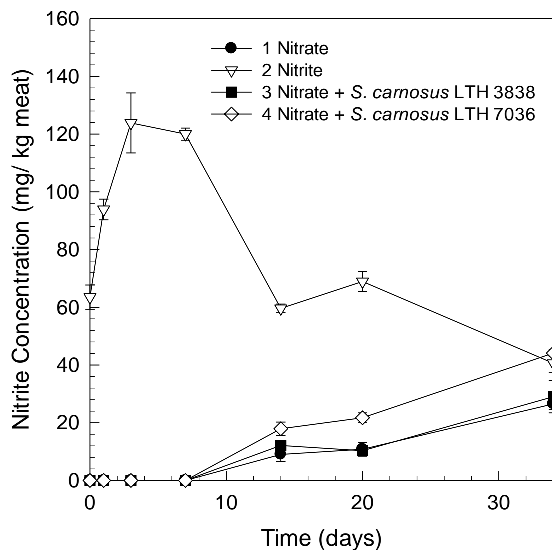
nitrite batch 2 without starter cultures. An increase of nitrite over the first 7 days during curing and a decrease at the beginning of drying and ripening.

Figure 2: Nitrate (a) and nitrite (b) development during process and storage of all raw ham batches.

(a)



(b)



The decrease of nitrite could be due to the process of color and aroma formation. Furthermore sensory analysis of the red color at day 20 showed, that batch 3 with the nitrate reductase active starter culture (4.9 ± 1.2) had the typical red to pink color like the nitrite control sample (5.0 ± 0.0). The nitrate control (5.7 ± 1.8) and the batch with low nitrate reductase active *S. carnosus* (5.8 ± 2.1) had a more red color like raw meat due to less nitrite concentration and lower amount of curing color pigments.

IV. CONCLUSION

The results showed that the application of starter cultures in raw ham by multi needle injection with nitrate is possible. Moreover, the trend of nitrite and nitrate is highly influenced by the presence of microorganisms. Therefore the selection of starter cultures due to their nitrate reductase activity plays an important role in the formation of curing agents and color formation in raw ham. Additionally, more studies on the color formation, color stability and aroma production should be carried out.

ACKNOWLEDGEMENTS

This research project was supported by the German Ministry of Economics and Energy (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project number AiF 17687 N. We would like to thank our student assistants Kevin Buchmann and Max Siessegger.

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